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REVIEW ARTICLE

Cellular and Molecular Mechanisms of Inflammation and Thrombosis

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In the last 20 years, the cellular and molecular mechanisms of inflammation and thrombosis have been characterised. These are essentially cell adhesion processes which are regulated by vascular endothelium. Many of the cell adhesion molecules and leucocyte chemoattractants expressed and generated at sites of inflammation have been sequenced and cloned. These inflammatory molecules work together in concert to mediate the adhesion between leucocytes, platelets and vascular endothelium which occurs during the occlusive, thromboembolic, reperfusion and septic complications of atherosclerotic and diabetic vascular diseases. This review aims to summarise our current understanding of the molecular basis of these disorders and the therapeutic implications.

Key Words: Cell adhesion molecules; Chemokines; Neutrophils; Inflammation; Atherosclerosis; Thrombosis.

Introduction

The human body reacts to injurious stimuli by mounting an inflammatory response. In its acute phase, transvascular shifts of fluid, plasma proteins and leucocytes occur in the microvasculature of the injured tissue. Vascular injury induces the activation of both platelets and coagulation proteins which work together to maintain haemostasis. These physiological processes are intended to neutralise the injurious stimulus, limit tissue injury and initiate healing and repair, and this was recognised by a surgeon, John Hunter, as early as the 19th century.[1]

The importance of vascular endothelium as a regulatory organ in immunity and haemostasis has become well established in the last two decades.[2] Normally, vascular endothelium is in an "unactivated" state. However, at sites of injury and inflammation, endothelium becomes locally "activated", either by the injurious stimulus itself, or by inflammatory cytokines generated in response to the injurious stimulus. Activated vascular endothelium expresses a number of membrane-bound pro-inflammatory molecules, in particular, leucocyte-endothelial cell adhesion molecules (CAM). It also secretes soluble factors which attract peripheral blood leucocytes and induce coagulation, thus resulting in the leucocyte recruitment and platelet thrombosis observed at sites of inflammation.

Inflammatory cytokines are small peptides secreted primarily by activated tissue macrophages and lymphocytes in response to injurious stimuli, such as endotoxin, immune complexes, physical and chemical injury. Interleukin-1β (IL-1β) and tumour necrosis factor α (TNF α) are the main cytokines that mediate the inflammatory response.[3] They have a wide range of actions on different cells but their main effects are to induce the acute-phase reaction and to activate vascular endothelium, leucocytes, platelets and fibroblasts, thus, initiating the cascade of vascular, cellular and humoral events which together comprise the inflammatory response.

In this review, we will focus on the role of non-lymphoid vascular endothelium, leucocyte-endothelial CAM, chemoattractants, leucocytes and platelets as the primary molecular and cellular components of the inflammatory response. The currently understood molecular mechanisms of atherogenesis and thrombosis will also be summarised. We will conclude by discussing possible therapeutic applications.

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Vascular Endothelium

Although a mere cellular monolayer, vascular endothelium contains the entire circulation and forms a vast surface area of interface between the components of the circulation (including leucocytes, platelets and plasma proteins) and all of the body's tissues. It performs a wide variety of functions including the regulation of vascular permeability and vascular tone, wound healing, immunity and haemostasis, thus, serving as a "gatekeeper" for the traffic of water and solutes, bioactive proteins and lipids, and peripheral blood leucocytes, between the circulation and interstitial tissues.

As a regulatory organ, vascular endothelium serves both sensory and effector functions, bearing receptors for a wide range of cytokines, chemotactic and vasoactive peptides and lipids, and CAM, as well as itself being able to express and elaborate many of these immunomolecules. Selective spatial and temporal expression of these molecules by vascular endothelium provides a means by which inflammatory and thrombotic processes may be regulated. Vascular endothelium continually senses chemical, physical and biomechanical alterations in its surroundings and transduces these into biological responses. For example, changes in blood flow have been shown to induce alterations in the synthesis of several vascular endothelial-dependent factors, such as prostacyclin, nitric oxide and intercellular adhesion molecule-1.[4–6]

Regulatory role in leucocyte traffic

In vivo studies in leucocyte-endothelial adhesion in the microcirculation and *in vitro* studies performed under flow conditions designed to simulate microvascular blood flow indicate that unactivated vascular endothelium is hypo-adhesive for leucocytes, by virtue of minimal expression of leucocyte-endothelial CAM.[7] However, stimuli such as endotoxin,[8] inflammatory cytokines,[3] histamine and thrombin[9] induce local endothelial activation or stimulation. Activated or stimulated vascular endothelium is hyperadhesive for leucocytes due to upregulated expression of CAM such as E-selectin,[10] intercellular adhesion molecule-1[11] and P-selectin.[9] Additionally, activated endothelial cells express and secrete chemotactic factors such as platelet-activating factor[12] and interleukin-8[13] which may activate adherent leucocytes and direct their migration across the endothelium and within the inflamed tissues.

Multiple inflammatory response genes which are regulated by cytoplasmic transcription factors, such as nuclear factor κB,[14] are induced during endothelial activation. Thus, endothelial activation is a process which occurs over a period of hours being dependent on gene expression and *de novo* protein synthesis. For example, the endothelial CAM, E-selectin, which is believed to be a "silent" gene under normal circumstances, is synthesised and expressed by activated endothelial cells, and becomes detectable between 1 and 24 hours after activation.[15] In contrast, thrombogenic mediators, such as histamine and thrombin, induce endothelial stimulation which occurs rapidly (within minutes) and independently of gene expression and protein synthesis. P-selectin is expressed transiently during endothelial stimulation.

Regulatory role in haemostasis

Unactivated endothelial cells are remarkable in being the only cells known to be actively antithrombotic. Firstly, they form an insulating barrier between thrombogenic subendothelial matrix components, and platelets and coagulation proteins in the circulation. Secondly, unactivated endothelial cells secrete prostacyclin and nitric oxide which inhibit platelet aggregation,[4,5] as well as protein S[16] and tissue plasminogen activator[17] which inhibit coagulation.

However, activated vascular endothelium is prothrombotic. It secretes tissue factor which triggers the coagulation cascade[18,19] and expresses increased levels of plasminogen activator inhibitor-1 which inhibits fibrinolysis.[20] Vascular injury may expose subendothelial matrix components such as collagen and von Willebrand factor (vWF) which promote and support platelet adhesion and aggregation.

Thus, at rest, vascular endothelium maintains blood in its fluid form but, in response to vascular injury, it becomes locally prothrombotic to seal off rapidly any breach in the integrity of the vasculature.

Leucocyte-endothelial Cell Adhesion Molecules

The histological hallmark of acute inflammation is the infiltration of interstitial tissues by peripheral blood leucocytes that consists in the early phases predominantly of neutrophils and, later on, mononuclear leucocytes. The ability of peripheral blood leucocytes to extravasate at sites of inflammation was described over a century ago,[21] but only in the past decade have the molecules responsible for leucocyte adhesion and

transendothelial migration (also called diapedesis, emigration) been identified and cloned, and their mechanisms of action defined.[22–24]

Leucocyte-endothelial cell adhesion molecules (CAM) are proteins expressed on the surface of leucocytes and platelets and the surface of vascular endothelium. They mediate the adhesive interactions between circulating leucocytes and endothelium leading to leucocyte recruitment into tissues, and between platelets and endothelium resulting in thrombosis. The role of CAM in leucocyte trafficking in health and disease has been established by *in vitro* experiments with cultured vascular endothelium and isolated leucocytes[25–27] and by *in vivo* experiments using animal models.[28,29] The generation of function-blocking monoclonal antibodies against individual CAM has enabled the various steps of leucocyte-endothelial adhesion to be characterised. More recently, experiments using genetically-engineered mutant mice lacking one or more CAM ("knockout mice") have further clarified their respective roles in leucocyte trafficking and immunity.[30–33] Furthermore, the importance of CAM in host defence is vividly illustrated by two rare autosomal recessively inherited diseases, leucocyte adhesion deficiency (LAD) Types I & II.[34,35] LAD I patients have deficient neutrophil diapedesis and phagocytosis due to molecular defects in their leucocyte integrins. Similarly, LAD II patients exhibit deficient neutrophil adhesion due to a metabolic defect in the synthesis of selectin ligands. LAD patients are markedly immunodeficient and suffer from severe, recurrent and often fatal bacterial infections.

To date, endothelial-leucocyte CAM have been classified according to structural homology into 4 families: the selectins, integrins, mucin-like CAM and the immunoglobulin superfamily (Table 1). The salient characteristics only of these CAM will be discussed below as more detailed reviews already exist.[22–24,36]

Selectins

Selectins are transmembrane glycoproteins expressed by leucocytes, and activated endothelium and platelets. Their primary function appears to be to mediate the initial attachment and rolling (discussed below) adhesive interactions that occur under pathophysiological conditions of blood flow between leucocytes and postcapillary venular endothelium at sites of inflammation.[24] There are three members of the selectin family.

CD62L or L-selectin ("L" for leucocyte) is constitutively expressed on the surface of most leucocytes.[37] Neutrophils and monocytes bear high surface levels of L-selectin that is lost or shed by proteolytic cleavage on contact with activated endothelium or chemoattractants.[27,38] A basal level of shedding appears to result in high levels of functionally active L-selectin in plasma[39] which may reach levels sufficient to partially inhibit leucocyte-endothelial adhesion.[40] However, *in vitro*, L-selectin shedding *per se* neither enhances nor inhibits neutrophil-endothelial adhesion and transmigration.[41]

CD62P or P-selectin ("P" for platelet) is normally not expressed but sequestered in intracellular storage granules in vascular endothelial cells (in Weibel–Palade bodies) and platelets (in α-granules).[9] Thrombogenic and inflammatory mediators such as histamine, thrombin, and leucotrienes C & D induce rapid translocation of P-selectin to the surface of platelets and vascular endothelial cells. *In vitro*, the expression of P-selectin is rapid and short lived (expressed then downregulated within minutes).

CD62E or E-selectin ("E" for endothelial) is expressed exclusively by activated vascular endothelial cells.[15] Induction of the E-selectin gene in cultured human umbilical vein endothelial cells by inflammatory stimuli, such as endotoxin, IL-1β or TNF-α, induces gene transcription and surface expression of the E-selectin protein which is detectable within an hour of endothelial activation. *In vitro*, peak E-selectin expression occurs around 4–6 h and becomes undetectable after 12–24 h. However, more prolonged expression of E-selectin may exist *in vivo* in chronic inflammatory diseases.[42,43]

The physiological counter-receptors of selectins have not been fully characterised, but they appear to be carbohydrate structures presented by glycoproteins on the surface of vascular endothelium, leucocytes and platelets.[24,44]

Mucin-like proteins

P-selectin glycoprotein ligand-1 (PSGL-1) is the best characterised selectin counter-receptor.[45,46] PSGL-1 is present on neutrophils, monocytes and lymphocytes and binds L-selectin, E-selectin and P-selectin.[47–49] Neutrophil–neutrophil adhesive interactions that occur during neutrophil aggregation as well as during initial attachment and rolling (discussed below) of neutrophils on activated vascular endothelium are mediated by PSGL-1–L-selectin bonds.[47,50] Current evidence suggests that another ligand for L-selectin may be expressed by activated non-lymphoid vascular

Table 1. Endothelial-leucocyte cell adhesion molecules.

Family	Members	Cluster designation	Cellular distribution	Counter-receptor/ligand
Selectins	E-selectin	CD62E	Endothelium	PSGL-1, ?other carbohydrate-bearing structure(s) on leucocytes
	L-selectin	CD62L	Leucocytes	PSGL-1, ?inducible carbohydrate-bearing structure(s) on endothelium
	P-selectin	CD62P	Endothelium, platelets	PSGL-1, ?other carbohydrate-bearing structure(s) on leucocytes
Mucin-like	PSGL-1	CD162	All blood leucocytes	E-, L- and P-selectin
β_1 integrins	$\alpha_4\beta_1$	CD29	Monocytes, lymphocytes	VCAM-1
β_2 integrins	LFA-1	CD11a/CD18	Leucocytes	ICAM-1, ICAM-2, ICAM-3
	Mac-1	CD11b/CD18	Monocytes, neutrophils	ICAM-1, fibrinogen
	p150,95	CD11c/CD18	Monocytes, neutrophils	?
β_7 integrins	$\alpha_4\beta_7$		Lymphocytes	VCAM-1
Ig	ICAM-1	CD54	Endothelium, leucocytes, epithelial cells, fibroblasts, other cell lines	LFA-1, Mac-1
	ICAM-2	CD102	Endothelium	LFA-1
	ICAM-3	CD50	Leucocytes	LFA-1
	VCAM-1	CD106	Endothelium, smooth muscle cells	$\alpha_4\beta_1$, $\alpha_4\beta_7$
	PECAM-1	CD31	Endothelium, leucocytes, platelets	PECAM-1

endothelium, although it has yet to be identified.[24,51] Other mucin-like proteins such as GlyCAM-1, CD34 and ESL-1, which bind selectins, have been described in animal models, but except for CD34 these have not been shown to be counter-receptors for selectins in humans.[24]

Leucocyte integrins

Integrins are transmembrane glycoproteins widely expressed on many cells.[34,52] They are heterodimers of an α subunit non-covalently linked to a β subunit and have been classified into subfamilies according to their β chain (8 β chains have been identified to date).

The main integrin subfamilies relevant to the role of leucocytes and platelets in inflammation and thrombosis are β_1, β_2, β_3 and β_7. β_1 integrins are expressed by a wide variety of vascular and non-vascular cells, the β_1 chain (CD29) combining with a variety of α chains. β_3 integrins or cytoadhesins are expressed by platelets, endothelial cells and smooth muscle cells (the β_3 chain being designated CD61). β_1 and β_3 integrins will be briefly discussed later in the context of platelets and thrombosis. However, the integrin subfamily most relevant to endothelial-leucocyte adhesion is the β_2 (CD18) or leucocyte integrin subfamily.

β_2 integrins are expressed exclusively by leucocytes. The β_2 integrin subfamily has three members all of which have CD18 as their common β chain: lymphocyte function associated antigen-1 (LFA-1) or $\alpha_L\beta_2$ (CD11a/CD18), Mac-1 or $\alpha_M\beta_2$ (CD11b/CD18), and p150,95 or $\alpha_X\beta_2$ (CD11c/CD18). To date, neutrophils are known to express only β_2 integrins while monocytes and lymphocytes express both β_1 and β_2 integrins.[36]

Unactivated neutrophils express high surface levels of LFA-1 and Mac-1 but minimal amounts of p150,95.[53] Mac-1 is also stored within intracellular peroxidase-negative granules.[54] On activation by inflammatory mediators such as chemoattractants, *in vitro*, the surface expression of Mac-1 is upregulated several-fold by translocation from cytoplasmic granules while that of LFA-1 remains relatively unchanged.[55–57] However, these newly-expressed Mac-1 molecules may not all be functionally competent, as further conformational change with the expression of activation neo-epitopes appears to be necessary for ligand binding. Expression of activation neo-epitopes which confer the ability to bind ligand has been described for both LFA-1 and Mac-1.[55,58]

LFA-1 counter-receptors include intercellular adhesion molecule-1 (ICAM-1), ICAM-2 and ICAM-3[59–61] while Mac-1 binds to ICAM-1, fibrinogen[55,62] and opsonic complement fragment (iC2b).[63] Mac-1 is also believed to mediate the binding of neutrophils to plastic and glass, and aggregation with other neutrophils.[53] The counter-receptor(s) of p150,95 are less certain, but it may be a complement receptor.[64]

Immunoglobulin superfamily

CAM belonging to the immunoglobulin (Ig) superfamily consist of cell surface proteins which are related structurally by possessing varying numbers of Ig-like domains. There are five members of the Ig-like family which mediate endothelial-leucocyte adhesion: intercellular adhesion molecule-1 (ICAM-1), ICAM-2, ICAM-3, vascular cell adhesion molecule-1 (VCAM-1) and platelet-endothelial cell adhesion molecule-1 (PECAM-1).

ICAM-1 (CD54) is widely expressed by many cell types including vascular endothelium, fibroblasts, epithelial cells, skeletal myocytes, and some leucocytes but not neutrophils.[65] It is constitutively expressed at low levels by vascular endothelium but its expression is upregulated several-fold by endotoxin and inflammatory cytokines.[11] Its counter-receptors include LFA-1, Mac-1, rhinovirus[66] and *Plasmodium falciparum*.[67] Soluble ICAM-1 detected in human plasma[39] has been proposed as a means by which the body modulates ICAM-1 mediated adhesion (by binding to its counter-receptors such as LFA-1 and Mac-1 on leucocytes). However, the concentrations of soluble ICAM-1 necessary to competitively inhibit ICAM-1–LFA-1 adhesion exceed those normally found in plasma and therefore, it is unlikely that soluble ICAM-1 would antagonise ICAM-1 mediated adhesion *in vivo*.[68]

ICAM-2 (CD102) is constitutively expressed by vascular endothelium and some mononuclear leucocytes. Its level of expression by vascular endothelium is not cytokine-inducible.[69] ICAM-3 (CD50), on the other hand, is highly expressed on leucocytes but not vascular endothelium.[61] Both ICAM-2 and ICAM-3 have been shown to bind LFA-1 but their role in leucocyte-endothelial adhesion remains uncertain.[60,61,69]

VCAM-1 (CD106) is expressed mainly by vascular endothelium but may also be expressed by vascular smooth muscle cells, dendritic cells and synovial cells.[70–72] VCAM-1 expression is focally upregulated by inflammatory and atherogenic stimuli such as IL-1β, interleukin-4 and lysophosphatidylcholine (a component of atherogenic lipoproteins).[73–75] Counter-receptors identified for VCAM-1 to date include the β_1 and β_7 integrins, VLA-4 ($\alpha_4\beta_1$) and $\alpha_4\beta_7$.[76,77] Neutrophils do not express β_1 and β_7 integrins and cannot bind to VCAM-1.

PECAM-1 (CD31) is constitutively expressed by vascular endothelium, neutrophils, monocytes, some lymphocytes and platelets.[23] Its expression on vascular endothelium is characteristic, being concentrated at intercellular junctions[78] where it mediates adhesion (CD31–CD31) between vascular endothelial cells. CD31 expression is not upregulated by inflammatory cytokines, although its distribution on the surface of endothelial cells may be altered by interferon γ and TNFα.[79,80] It also mediates adhesion between myeloid cells (neutrophils and monocytes) and platelets as well as between myeloid cells and vascular endothelium. It appears to be important in mediating neutrophil and monocyte transmigration between intercellular junctions of normal and activated vascular endothelium.[81,82] Recently, CD31 has been shown to undergo adhesion to the integrin $\alpha_V\beta_3$.[83]

Platelet glycoproteins

Platelets are crucial to the body's normal inflammatory response because of their ability to form haemostatic platelet plugs very rapidly at sites of vascular injury. They also play an important role in initiating healing processes, by virtue of the growth and chemotactic factors stored in their alpha granules and released on activation. The surfaces of these tiny non-nucleated cells are densely decorated with β_1 and β_3 integrins and other platelet glycoproteins (gp) which mediate the two platelet reactions that are crucial for normal haemostasis, namely, platelet adhesion and platelet aggregation. Platelets adhere to subendothelial matrix proteins exposed by vascular injury (platelet adhesion). Then, on activation, by the process of platelet adhesion and/or by agonists such as adenine diphosphate (ADP), adrenaline and thrombin, more platelets are recruited and begin to adhere to each other (platelet aggregation) and to form a haemostatic plug onto which fibrin may be deposited. Leucocyte recruitment mediated by P-selectin appears to be involved in this process of fibrin deposition.[84]

Platelet adhesion is mediated by $\alpha_2\beta_1$ (binds collagen), $\alpha_5\beta_1$ (binds fibronectin), $\alpha_6\beta_1$ (binds laminin) and $\alpha_{IIb}\beta_3$ (also called, gpIIb/IIIa) as well as other glycoproteins, such as gpIb (binds vWF).[85,86] Platelet aggregation, however, is mediated exclusively by the β_3 integrin, $\alpha_{IIb}\beta_3$ (gpIIb/IIIa). gpIIb/IIIa binds to a number of soluble proteins including fibrinogen and vWF but becomes functional (by "uncovering" of ligand binding sites which lie hidden on unactivated platelets) only on platelet activation.[87,88] Fibrinogen and vWF form crossbridges between gpIIb/IIIa molecules on adjacent platelets, thereby resulting in platelet aggregation. Blockade of gpIIb/IIIa totally inhibits platelet aggregation.

Chemoattractants

The proces of "chemotaxis" may be defined as the unidirectional locomotion of a cell in response to a

Table 2. Leucocyte chemoattractants.

Family	Member	Source	Target cell(s)
"Classical"	C5a	Plasma protein cleavage product	Monocyte, neutrophils, basophils, eosinophils
	fMLP	Bacteria	Monocytes, neutrophils
	PAF	Leucocytes	Monocytes, neutrophils, eosinophils
	LTB_4	Leucocytes	Neutrophils
C-X-C chemokines	IL-8	Most somatic cells	Neutrophils, basophils
	GRO-α, -β, -γ	Most somatic cells	Neutrophils, basophils
	IP-10	Most somatic cells	Monocytes
	PF4	Platelets	Monocytes, neutrophils
C-C chemokines	MCP-1, -2, -3	Most somatic cells	Monocytes, basophils, specific lymphocyte subsets
	MIP-1α	Most somatic cells	Monocytes, basophils, eosinophils, specific lymphocyte subsets
	MIP-1β	Most somatic cells	Monocytes, specific lymphocyte subsets
	RANTES	Most somatic cells	Monocytes, basophils, eosinophils, specific lymphocyte subsets

concentration gradient of a chemoattractant substance. Leukocytes, in particular, have long been observed to exhibit chemotaxis.[21] Chemical substances or microorganisms placed at extravascular sites, such as the anterior ocular chamber or the peritoneal cavity, induce the accumulation of large numbers of peripheral blood leucocytes at these sites as part of an inflammatory response.

To date, a wide range of chemotactic molecules have been identified which attract and activate leucocytes.[89–91] Furthermore, specific chemotactic receptors found on the leucocyte surface have now been cloned.[92,93] Presumably, by using these receptors leucocytes are able "to sense" small concentration differences of chemotactic substances across their diameter and then crawl in the direction of increasing concentration.[94]

"Classical" chemoattractants

A variety of bioactive peptides and lipids of endogenous and exogenous origin were identified in the 1970s and found to be leucocyte chemoattractants. Activated neutrophils produce an oxidised phospholipid leucotriene B_4 (LTB_4),[95] as well as an ether phospholipid, platelet-activating factor (PAF),[96,97] both of which are chemotactic for neutrophils. PAF-like molecules are also generated by activated vascular endothelium in a membrane-bound form.[98] PAF, apart from being a leucocyte chemoattractant, has diverse biological activities including enhanced adhesion of platelets and leucocytes to endothelium, vasoconstriction and bronchoconstriction.[96,99] Both C5a anaphylatoxin, a plasma peptide generated by complement activation, and its cleavage product, $C5a_{des\ arg}$, are potent chemoattractants for neutrophils,[100] basophils, eosinophils and monocytes. fMLP, a bacterial oligopeptide, has also been shown to induce chemotaxis in neutrophils and monocytes.[101] It should be noted that these chemoattractants neither exhibit specificity of chemoattraction for any particular leucocyte subset nor are their biological activities confined to inducing leucocyte chemotaxis alone. PAF, as its name suggests, is a potent activator of platelets and leucocytes as well as having vasoactive properties.[99]

Chemokines

Chemokines (contraction of *chemo*tactic cyto*kines*) are a family of small pro-inflammatory peptides with potent leucocyte chemoattractant and activating properties.[90,102] To date, they have been classified into two main subfamilies according to amino acid sequence, their gene locus and the leucocyte subset on which they primarily exert chemotactic activity (Table 2). This specificity of chemoattraction of a particular leucocyte subset is a key feature of chemokines, in contrast to "classical" chemoattractants discussed above.

Chemokines are mostly inducible molecules, that is, produced and secreted only on cellular activation by endotoxin or inflammatory cytokines or in response to agents such as crystals and viruses.[90] Induction of secretion of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) has been described in a wide variety of cells while a small group of C-X-C chemokines have only been found preformed and stored in platelet granules. Apart from chemotaxis, chemokines also induce leucocyte activation. IL-8 and MCP-1 induce degranulation and the respiratory burst in neutrophils and monocytes respectively.[103,104] Additionally, while IL-8 induces PAF secretion by neutrophils,[105] MCP-1 induces IL-1 and IL-6 secretion by monocytes.[106]

C-X-C (or α) chemokines are so-called because they have an intervening amino acid between the first two of the four conserved cysteines at their N-terminus. They are encoded for on chromosome 4.[107] C-X-C chemokines include IL-8, epithelial-derived neutrophil attractant-78 (ENA-78), growth-related cytokine-α (GRO-α), interferon γ-inducible protein-10 (IP-10) and platelet factor-4 (PF4). C-X-C chemokines (IL-8, GRO, ENA-78) are primarily neutrophil chemoattractants. However, IP-10 attracts monocytes but not neutrophils[108] while PF4 can attract both neutrophils and monocytes.[109] IL-8 can also attract basophils[110] and by an indirect mechanism, T-cells.

C-C (or β) chemokines, on the other hand, do not have an intervening amino acid between their first two amino-terminal cysteine residues. They are encoded for on chromosome 17[109] and are primarily monocyte and T-cell chemoattractants.[111,112] C-C chemokines include MCP-1, MCP-2, MCP-3, monocyte inflammatory protein-1α (MIP-1α), MIP-1β and RANTES (regulated on activation, normal T expressed and secreted).

Mechanisms of chemoattractant function

In vitro assays of chemotaxis[113,114] suggest that a "diffusion barrier" located between leucocytes and the "reservoir" of chemoattractant substance is necessary to establish a concentration gradient. Leucocytes sense and crawl "up" this gradient in the direction of increasing concentration. In contrast, leucocytes placed in an environment of uniform chemoattractant concentration undergo enhanced random and directionless movements called "chemokinesis".[115]

In vivo, vascular endothelium, the vessel wall, and the interstitial tissue spaces may serve the function of being a "diffusion barrier" that establishes a concentration gradient. Blood flow, by dilution and fluid motion, would actively tend to lower the concentration of chemoattractant in the intravascular compartment. Moreover, red blood cells and venous endothelium bear large numbers of a "scavenger receptor" for chemokines,[116,117] which would tend to lower the concentration of functional chemoattractant within the circulation. However, these factors are absent in the extravascular/interstitial compartment where chemoattractants may accumulate. High levels of IL-8, for example, have been found sequestered in extravascular tissue compartments such as infected joint and pleural cavities.[118–120] IL-8 has been reported to be secreted in a polarised fashion into the extravascular compartment.[121]

In summary, it appears that chemoattractants concentrated in extravascular tissue spaces induce leucocyte recruitment, while high levels of intravascular chemoattractant tend to attenuate leucocyte extravasation.[102,122]

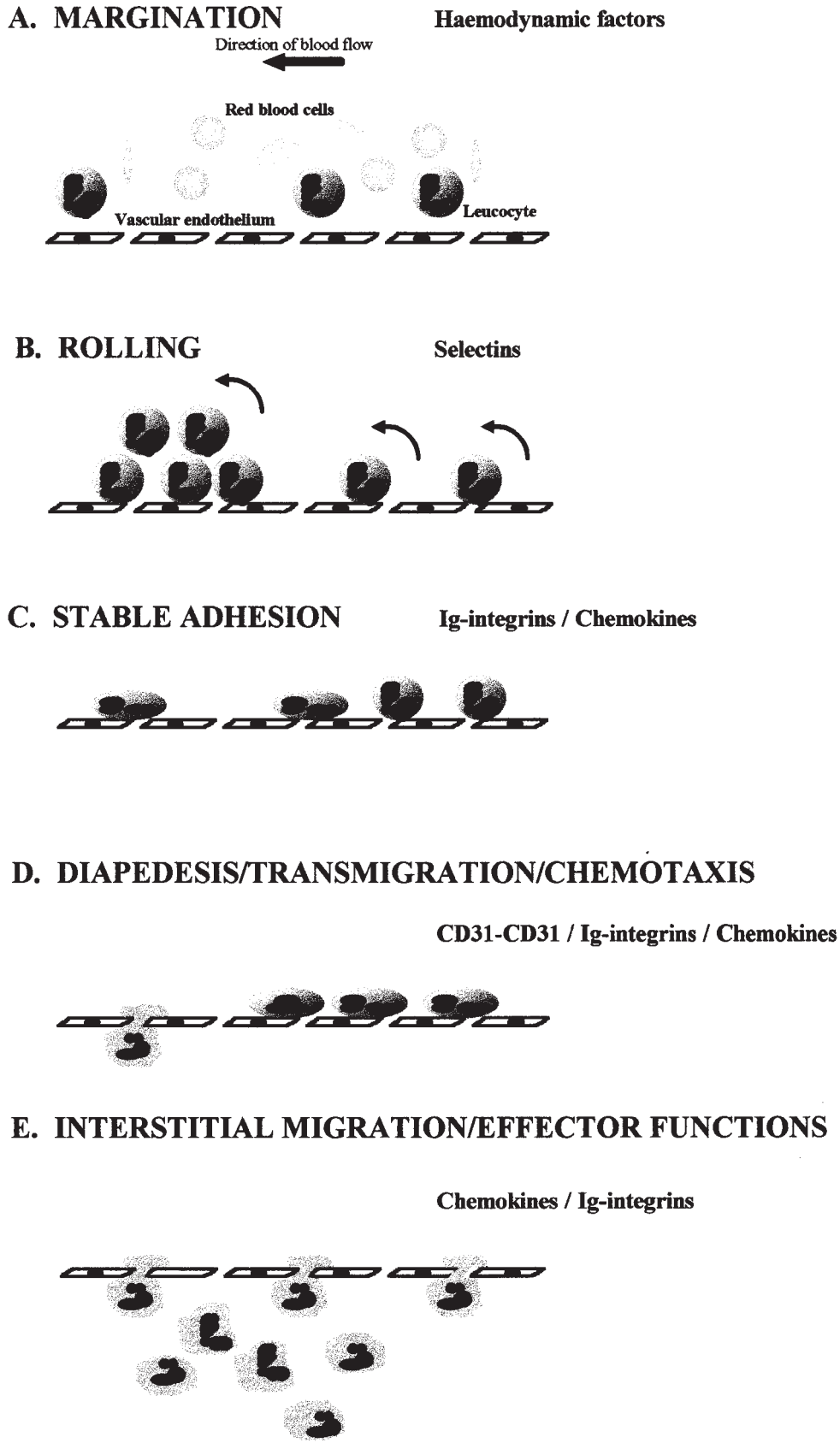
The Leucocyte-endothelial Adhesion Cascade

The recruitment of leucocytes is precisely regulated by vascular endothelium, which, on activation, presents an adhesive surface to circulating leucocytes. The process of adhesion and diapedesis then involves sequential binding of different CAM to their counter-receptors in a so-called "adhesion cascade". Generally, leucocyte CAM are expressed constitutively, while vascular endothelium express CAM on activation. *In vivo* studies, particularly using intravital microscopy, indicate that leucocyte adhesion and diapedesis mostly occurs in post-capillary venules rather than in arterioles or capillaries.[123,124] This may, in part, be due to higher blood flow rates in arterioles, but may also be related to intrinsic differences in the venular and arteriolar endothelial expression of CAM necessary for adhesion under conditions of flow.

The individual steps of the adhesion cascade became apparent from direct observation of the dynamics of leucocyte-endothelial adhesion, by both intravital videomicroscopy[28,29] as well as by using *in vitro* flow systems in which the pathophysiological and hydrodynamic conditions of leucocyte adhesion under fluid flow at the luminal surface of vascular endothelium were simulated.[27,125] The five main steps of the adhesion cascade are: (1) random contact and margination, (2) initial attachment (or tethering) and rolling, (3) stable arrest and adhesion, (4) transmigration (or diapedesis), (5) interstitial migration and carrying out effector functions (Fig. 1).

Margination (Fig. 1A)

This first step in leucocyte-endothelial adhesion *in vivo* is a purely random and haemodynamic phenomenon that does not involve the formation of any cell adhesion bonds. Normally, blood flowing through postcapillary venules may be observed to form a central cellular zone consisting of red blood cells and leucocytes and a peripheral cell-free zone.[126] At sites of inflammation, they tend to be pushed away from the centre stream towards the endothelial surface (marginated) by a number of haemodynamic factors.


A. MARGINATION
Haemodynamic factors
Direction of blood flow
Red blood cells
Vascular endothelium
Leucocyte
B. ROLLING
Selectins
C. STABLE ADHESION
Ig-integrins / Chemokines
D. DIAPEDESIS/TRANSMIGRATION/CHEMOTAXIS
CD31-CD31 / Ig-integrins / Chemokines
E. INTERSTITIAL MIGRATION/EFFECTOR FUNCTIONS
Chemokines / Ig-integrins

Smaller-sized red blood cells travelling at greater velocities tend to marginate leucocytes.[127] Leucocytes from smaller tributaries feeding into the side of larger venules are pushed by the prevailing blood flow in the larger vessel towards its sidewalls.[123] During inflammation, acute phase proteins in the circulation increase the viscosity of the blood and may induce the formation of erythrocyte aggregates. This, along with the slowing of blood flow in the microcirculation at sites of inflammation due to vasodilation, will also tend to marginate leucocytes.[128]

The release of vasodilators, e.g. histamine and prostaglandins, during the inflammatory response may increase blood flow and, therefore, the delivery of leucocytes to sites of inflammation. In the presence of tissue injury, upregulated CAM expression may result in adhesive interactions between the marginated leucocytes and the hyperadhesive vascular endothelial surface, thus initiating the adhesion cascade.

Rolling (Figure 1B)

Leucocytes in postcapillary venules at sites of inflammation may be observed to decelerate and "roll". This is the resultant of transient adhesion bonds being repeatedly formed and broken, between endothelial and leucocyte CAM under the prevailing hydrodynamic forces of fluid flow.

This "rolling" behaviour of leucocytes contacting the luminal surface of activated vascular endothelium is mediated primarily by the selectins,[24,129] although VCAM-1 has also been shown to mediate leucocyte rolling.[130] The shear forces at the luminal surface of postcapillary venules are around 1–5 dynes/cm^2 [131,132] and require the formation of selectin-mediated bonds to "capture" marginated leucocytes being swept along by blood flow. At lower flow rates (shear rate ≤ 0.6 dynes/cm^2), L-selectin appears to become less efficient at mediating rolling,[133] and then other adhesion mechanisms, such as β_2 integrin-ICAM-1, may function in mediating initial attachment and rolling.[125,134]

Neutrophils have been shown to undergo rolling on both activated vascular endothelium as well as on other neutrophils already stably adherent to the endothelial surface *in vitro*.[50,135] Neutrophil–neutrophil rolling and aggregation may be a means of amplifying the rate of recruitment of neutrophils at sites of inflammation.[50,136] Free-flowing neutrophils may be seen to tether to activated vascular endothelium and then to form linear aggregates and discrete foci of rolling neutrophils.[27,50] These neutrophil–neutrophil adhesive interactions are mediated by L-selectin binding to PSGL-1. Leucocytes carry both L-selectin as well as PSGL-1 preferentially on the tips of their microvilli, ideally placed for mediating initial attachment and rolling on other neutrophils or vascular endothelium.[46,137]

In summary, a selectin-mediated step is required for leucocyte-endothelial adhesion to occur and culminate in successful leucocyte recruitment, under pathophysiological conditions of fluid flow.

Stable arrest and adhesion (Figure 1C)

Rolling leucocytes having decelerated begin to form more stable adhesion bonds and, thus, "arrest" in stationary positions on the endothelial surface despite the prevailing shear forces of fluid flow. A necessary prerequisite for this step to occur successfully appears to be integrin activation. Chemokines (e.g. IL-8) and other chemoattractants, such as PAF,[26] are generated at inflammatory sites.[138] Leucocytes rolling on activated endothelium may be exposed to chemokines/chemoattractants or other stimuli which activate the leucocyte integrins rendering them competent to bind ICAM-1 and VCAM-1. Thus, stable arrest in neutrophils and monocytes is mediated by β_2 integrin–ICAM-1 adhesion.[27,29] Additionally, mononuclear leucocytes also employ β_1 and β_3 integrins to bind to VCAM-1[27] and stably adhere to activated vascular endothelium.

Transendothelial migration/diapedesis (Figure 1D)

Stably adherent leucocytes then begin to flatteen and spread on the luminal surface and extend pseudopods

Fig. 1 (*opposite*) (A) Margination is largely a haemodynamic phenomenon and does not depend on any adhesion bonds. (B) Initial attachment and rolling is mediated by selectins (e.g. L-selectin) binding their counter-receptors (e.g. PSGL-1). Neutrophils roll and decelerate on both activated endothelium as well as other adherent neutrophils. (C) Stable adhesion is mediated by adhesion between integrins (e.g. Mac-1 and $\alpha_4\beta_1$) present on the leucocyte surface and Ig (e.g. ICAM-1 and VCAM-1) on the surface of activated endothelium. (D) Transendothelial migration is mediated by CD31–CD31 adhesion between stably adherent leucocytes and endothelium as well as integrin–Ig bonds. Chemokines may promote diapedesis by activating leucocyte integrins or inducing chemotaxis. (E) Chemokines sequestered in extravascular tissue spaces may direct the interstitial migration of transmigrated leucocytes and induce leucocyte activation. Extravasated leucocytes may in turn generate more chemoattractants, thereby amplifying the recruitment process.

towards intercellular borders. This process which is mediated by leucocyte integrins engaging ICAM-1 and/or VCAM-1 appears to involve the coordinated formation and breaking and then re-formation of adhesion bonds as the leucocyte "crawls" across the endothelial surface towards the intercellular border without being swept away by fluid flow. At the intercellular border leucocytes extend pseudopods between endothelial cells and transmigrate into the extravascular spaces. The process of neutrophil and monocyte transmigration or diapedesis between the intercellular junctions of normal and activated vascular endothelium has been shown to be mediated in part by both integrin–ICAM-1 or integrin–VCAM-1 as well as CD31–CD31 adhesive interactions.[25,81,82] The relative importance of each mechanism remains to be further clarified for different leucocyte subsets. For example, some leucocytes (memory T-cells) do not express CD31 and yet are able to transmigrate successfully.[139] Chemoattractants may promote diapedesis by inducing chemotaxis or by integrin activation.

Interstitial migration and effector functions (Figure 1E)

At sites of inflammation, chemoattractants are generated and then sequester in interstitial and extravascular tissue spaces, being secreted by tissue macrophages and non-vascular cells such as fibroblasts.[119,140–142] Chemokines entering the circulation are carried away by blood flow and become functionally inactive by binding to scavenger receptors found on the surface of red blood cells and venular endothelium.[143] Functionally active chemokines and other chemoattractants thus tend to localise in extravascular tissue compartments and from there induce chemotaxis and activation of extravasated leucocytes, which then are induced to carry out their respective effector functions such as cytokine secretion, phagocytosis and degranulation.

Mechanisms of Amplifying the Inflammatory Response

A number of mechanisms exist which seem to both regulate and amplify the inflammatory response. L-selectin–PSGL-1-mediated adhesion and aggregation between neutrophils may be a means of localising and amplifying the numbers of neutrophils "captured" to adhere to activated endothelium at sites of inflammation.[50] Inflammatory mediators may be sequentially generated by different cells. For example, C5a produced during myocardial ischaemia-reperfusion injury mediates recruitment of neutrophil which in turn, on activation, may generate IL-8, PAF and LTB_4, thus inducing further neutrophil recruitment.[144,145] Similar mechanisms may exist for monocytes and other leucocytes.[146] Furthermore, there appears to be a system of networking between different cells via cytokines and other chemoattractants to amplify the inflammatory response. During Gram-negative pulmonary sepsis, alveolar macrophages, on activation by endotoxin, secrete inflammatory cytokines which then act on pulmonary fibroblasts to generate IL-8, while endotoxin alone did not induce significant IL-8 secretion by pulmonary fibroblasts.[141]

Endothelial-mediated Mechanisms of Atherosclerosis

The importance of atherosclerosis as a leading cause of morbidity and mortality is undisputed. Atherosclerosis is thought to be an excessive inflammatory-fibroproliferative response to chronic injury in susceptible arteries.[147] It appears to be the result of complex interactions between arterial endothelium and smooth muscle cells, and surrounding physical and biochemical factors such as circulating leucocytes and platelets, proteins and lipids carried in plasma and haemodynamic forces.[2]

The earliest anatomical lesion of atherosclerosis is a subendothelial accumulation of lipid-laden macrophages called the "fatty streak". An even earlier cellular event during atherogenesis is the increased adherence of peripheral blood monocytes to the endothelium of large arteries at sites which later develop fatty streaks.[148] In a rabbit model of atherogenesis, recruitment of peripheral blood monocytes is preceded by focal VCAM-1 expression.[149] VCAM-1, expressed by activated arterial endothelium, mediates monocyte-endothelial adhesion,[27] and may be one of the earliest molecular markers of atherosclerosis. At these sites, fatty streaks develop and later progress into fibro-fatty atherosclerosis plaques containing monocytes and T-lymphocytes.[150] Other CAM, namely, ICAM-1, CD31, and P-selectin, have also been shown to be expressed by human atherosclerotic plaques[151,152] and each of these is capable of mediating leucocyte-endothelial adhesion.[27,82,153]

Hyperlipidaemia *per se* results in a number of pro-atherogenic changes in the vessel wall. It induces endothelial activation and the expression of MCP-1, a monocyte chemoattractant.[154] Hyperlipidaemia also stimulates monocyte production of IL-1β,[155,156] which in

turn induces further endothelial activation and MCP-1 expression. IL-1β may also act as a growth factor for smooth muscle cells.[157] Furthermore, cytokine activation of endothelium and smooth muscle cells has been shown to induce the expression of macrophage colony stimulating factor,[158] a cytokine involved in the growth and maturation of monocyte-macrophages. Diabetics develop premature and florid atherosclerosis and a possible mechanism for this may involve the formation of advanced glycation endproducts in the vascular wall in response to chronic hyperglycaemia. These advanced glycation endproducts have been shown to promote monocyte adhesion and chemotaxis.[159,160] Monocyte-macrophages, which are present at all stages of atherogenesis, may in turn release a number of inflammatory cytokines and growth factors involved in the progression of the atherosclerotic plaque.[159]

Therapeutic Applications

While endothelial-leucocyte adhesion and diapedesis are necessary for normal host defence, there exist a number of inflammatory diseases in which excessive or inappropriate leucocyte recruitment contributes to tissue damage. For example, in atherosclerosis, mononuclear leucocytes accumulate in the arterial wall and secrete inflammatory cytokines and growth factors which promote plaque progression. Although the pathogenesis of atherosclerosis is not fully understood, it is believed to be an inflammatory response to injurious stimuli.[159] Therefore, the ability to inhibit the recruitment of mononuclear leucocytes may be a way of modulating plaque progression. Similarly, restoration of blood flow following a period of limb or organ ischaemia is characterised by recruitment and activation of neutrophils which are believed to induce postischaemic tissue injury during reperfusion. Platelet thrombosis occurs by two cell adhesion processes (platelet adherence and aggregation), both of which are mediated by platelet integrins and glycoproteins. All these disease processes depend on the adhesion and aggregation of leucocytes and platelets to vascular endothelium mediated by CAM and chemoattractants.

Potential molecular targets

Knowledge of the cellular and molecular mechanisms of leucocyte recruitment and platelet thrombosis affords the possibility of pharmacologically modulating these cell adhesion processes and a number of molecular sites present themselves as being potential targets (Table 3). Antagonism of the cytokines which drive the inflammatory response may reduce the severity of tissue damage locally and dampen undesirable systemic effects. Disruption of some or all of the steps of the endothelial-leucocyte adhesion cascade, by blockade of CAM and/or chemoattractants, may be a means of controlling leucocyte recruitment in inflammatory diseases. Instead of blockade at the level of extracellular protein, it may be possible to antagonise the expression of one or more inflammatory response genes at the cytoplasmic transcription factor or proteasomal level.[161,162] Finally, endothelial-leucocyte adhesion may be modulated by reducing the systemic leucocyte count by the use of extracorporeal leucocyte filters.

Possible limitations

The temporal and spatial patterns of expression of CAM *in vivo* in various disease states have yet to be fully characterised, and, therefore, it may not be possible at present to effectively block a particular CAM in a specific and timely fashion. Furthermore, the existence of multiple or redundant mechanisms of leucocyte recruitment in particular organs and diseases may necessitate the simultaneous blockade of multiple adhesion pathways. For example, in the pulmonary microcirculation, CD18-dependent and CD18-independent mechanisms of leucocyte recruitment appear to operate.[163]

Monoclonal antibody (mAb) therapy has certain theoretical limitations. mAb generated using animals may induce sensitisation or contain endotoxin or virus contamination. The Fc portion of mAb may mediate complement activation and non-specific neutrophil binding once the $F(ab')_2$ portion has bound to the endothelial target antigen. However, while $F(ab')_2$ fragments are able to bind and block the target antigen, they have brief plasma half-lives being cleared relatively rapidly from the circulation. Additionally, in animal studies, mAb have been shown not to be efficacious in reducing leucocyte recruitment unless their efficiency of blockade exceeded 90%.[164] Once a few neutrophils adhere, these appear able to recruit other neutrophils, presumably by amplification mechanisms (see above).

A theoretical concern of blockade of β_2 (CD18) integrins is the possibility of inducing an immunocompromised state and uncontrolled sepsis by interfering with the ability of neutrophils to extravasate and kill bacteria at sites of infection. Animal

Table 3. Molecular targets in the modulation of inflammation and thrombosis.

Target molecule	Function	Pharmacological agent
Inflammatory cytokines	Endothelial and leucocyte activation	Monoclonal antibody Soluble receptor antagonist
Selectins	Leucocyte-endothelial initial attachment and rolling	Monoclonal antibody Soluble selectin or ligand Synthetic carbohydrate
β_2 integrins Ig	Stable adhesion of leucocytes and diapedesis	Monoclonal antibody Soluble CAM Soluble counter-receptor
gpIIb/IIIa Platelet integrins	Platelet aggregation Platelet-endothelial adhesion	Monoclonal antibody
Chemoattractants Chemokines	Integrin activation and leucocyte recruitment	Monoclonal antibody Soluble receptor Synthetic receptor antagonist
Transcription regulation of inflammatory response genes	mRNA synthesis and protein synthesis of inflammatory response gene product e.g. E-selectin	Proteasome inhibitors Antisense oligonucleotides

studies have yielded conflicting results as to whether CD18-blockade increased septic complications.[165,166]

Ischaemia-reperfusion injury

Tissue ischaemia may occur in a variety of clinical situations including acute vessel occlusion by thromboembolism, trauma or surgical cross-clamping of major vessels, compartment syndrome, autogenous muscle transplantation for wound coverage and solid organ transplantation. During ischaemia, cellular injury is caused by a reduction in the supply of oxygen and nutrients as well as inadequate clearance of products of metabolism. Survival of ischaemic tissue is, therefore, dependent on timely and sufficient restoration of its blood supply (reperfusion). However, beyond a certain period of ischaemia, reperfusion may itself result in additional damage of postischaemic tissues and an accelerated rate of necrosis despite adequate restoration of blood flow.[167,168] Neutrophil infiltration is characteristic of the inflammatory response to reperfusion following ischaemia.[168,169] The pathogenesis of reperfusion injury is complex but is believed to be due in part to neutrophil-mediated damage.[168,170–172] Both CAM and chemoattractants have been shown to mediate neutrophil recruitment in reperfusion injury.[144,164,172] Blockade of CAM and chemoattractants in numerous animal models of ischaemia and reperfusion in different organs appeared to reduce the extent of tissue damage during reperfusion.[144,164,168,172] These findings remain to be confirmed in clinical studies.

Platelet thrombosis, restenosis and thrombolysis

The surface exposed by vascular endothelial injury following balloon angioplasty or ulceration of atherosclerotic plaque is highly thrombogenic. Platelets adhere to subendothelial matrix proteins such as collagen and fibronectin, and then become activated and degranulate, releasing coagulation proteins, calcium ions (necessary for coagulation), ADP (mediates platelet aggregation), and chemokines (e.g. PF4). Activated platelets may also secrete thromboxane A_2, a potent vasoconstrictor and stimulus of platelet aggregation.

Low-dose aspirin inhibits the synthesis of thromboxane A_2 by irreversibly acetylating the enzyme cyclooxygenase. As platelets are anuclear cells unable to synthesise new proteins, they cannot manufacture new enzyme during their short lifespan of just over a week. In contrast, prostacyclin (an inhibitor of platelet aggregation) production by endothelial cells is relatively unaffected by low doses of aspirin. Thus, low dose aspirin tips the balance of prostaglandin metabolism towards inhibition of platelet aggregation.[173] Ticlodipine and clopidogrel are novel antiplatelet agents which are more potent than aspirin.[174] They are inactive *in vitro*, but *in vivo*, after hepatic metabolism into their active moieties, they produce irreversible platelet inhibition by selectively blocking ADP receptors and inhibiting fibrinogen binding of gpIIb/IIIa.[175]

Following balloon angioplasty of atherosclerotic vessels, damage to the intima may induce platelet activation and aggregation and thrombosis resulting in acute thrombotic occlusion (termed "abrupt closure"). Activated platelets may also be a source of growth

factors which mediate smooth muscle cell proliferation that characterises both progression of atherosclerotic plaques as well as late restenosis of angioplastied lesions. A chimeric mAb F(ab')$_2$ fragment against gpIIb/IIIa (c7E3; trade name ReoPro) has been shown in clinical trials to reduce the incidence of acute ischaemic complications due to platelet thrombosis following coronary angioplasty and atherectomy.[176,177] Unfortunately, the rate of restenosis has not been consistently reduced. However, blockade of platelet thrombosis with anti-gpIIb/IIIa mAb following coronary angioplasty significantly reduced the rates of death, myocardial infarction and need for further revascularisation.[178] Blockade of platelet thrombosis may also prove to be useful in selected cases of peripheral e.g. infrainguinal angioplasty.

It should be noted that platelets appear to have both profibrinolytic as well as antifibrinolytic activity. Both streptokinase and tissue-plasminogen activator induce platelet activation,[179] and platelet-rich thrombi appear to be more resistant to lysis.[180] On the other hand, thrombolysis may be accelerated in the presence of platelets by binding plasminogen via a gpIIb/IIIa-dependent mechanism, thus generating high local concentrations of plasmin.[181] The concomitant use of antiplatelet agents during thrombolysis seems to expedite reperfusion and may lower the dose of thrombolytic agent required to attain reperfusion, and so reduce the risk of haemorrhage.[86] Further studies are necessary to determine the effects of platelets and antiplatelet therapy during thrombolysis in different clinical settings.

Conclusions

Thus, the pathogenesis of a number of vascular diseases and their complications depend on cell adhesion processes mediated by CAM and chemoattractants and occur as vascular endothelial cells respond to injurious stimuli. The continued understanding of these molecular processes will allow the development of therapeutic strategies in the future to treat and arrest the progression of these diseases by modulating leucocyte-endothelial and platelet-endothelial adhesion.

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